

AMENDMENTS TO THE SPECIFICATION

Please amend the first sentence of the application a page 1 as follows:

This application is a continuation of application serial no. 09/376,911 filed on August 18, 1999, now abandoned, which claims benefit of Provisional application serial no. 60/097,120 filed on August 19, 1998.

Please replace the paragraph starting on page 6, line 18 of the specification with the following amended paragraph:

Capsular polysaccharides (CPS) amenable to base extraction are those polysaccharides that lack any base-labile substituent that cannot be replaced, such as O-acetyl groups critical to immunogenicity. Other capsular polysaccharides amenable to base extraction are those lacking a phosphodiester bond and those lacking 4-linked uranic acid residues.

Please replace the paragraph starting on page 8, line 30 of the specification with the following amended paragraph:

In one embodiment, the method comprises adding an acryloylating reagent to N-acryloylate an N-deacetylated polysaccharide or oligosaccharide. Examples of acryloylation reagents include but are not limited to acryloyl chloride, acryloyl anhydride, acrylic acid and a dehydrating agent such as dicyclohexylcarbodiimide (DCC), CH_2CHCOCN the like, used in excess at a concentration of about 1 M. In a method of N-acryloylation of an N-deacetylated polysaccharide, the pH is adjusted and maintained at about 9 to about 11, preferably about pH 10 during the reaction. The temperature during reaction is about 2°C to about 8°C, preferably about

4°C. The reaction is carried out over a period of about 1 hour. The resulting N-acryloylated polysaccharide or N-acryloylated oligosaccharide is at least about 95% acryloylated or greater.

Please replace the paragraph starting on page 17, line 15 of the specification with the following amended paragraph:

To increase its solubility the polysaccharide was first partially depolymerized by sonication. 200 mg of *Pneumococcal polysaccharide* type 14 (Lot NO 2020510, American Type Culture Collection) was dissolved in 20 ml of PBS and sonicated for 4 hours at 0°C with a Branson ~~Sonifier~~ SONIFIER® Model 450. The resulting polysaccharide was dialyzed and lyophilized and then sized through a ~~superdex~~ SUPERDEX® 200 column equilibrated with phosphate buffered saline (PBS). Peak fractions were pooled and then dialyzed against d.i. water with ~~Spectra/Por®~~ SPECTRA/POR® Membrane MWCO:3,500. A yield of 157.5 mg solid was obtained after lyophilization. The sonicated polysaccharide had an average molecular weight of about 50,000 as measured by SEC-MALLS with the ~~miniDAWN~~ MINIDAWN® (Wyatt Technology Corp., Santa Barbara, Calif.).

Please replace the paragraph starting on page 17, line 26 of the specification with the following amended paragraph:

100 mg of sized type 14 pneumococcal polysaccharide was dissolved in 10 ml of 2N NaOH and then 10 mg of NaBH₄ was added to the reaction mixture. This mixture was heated at 100°C for one hour and then cooled to room temperature. The N-deacetylated component was dialyzed against d.i. water with a ~~Spectra/Por®~~ SPECTRA/POR® Membrane Membrane MWCO:3,500 and lyophilized to give 84 mg of white solid. The N-deacetylated polysaccharide

was analysed by H^1 -NMR at 500 MHz and was found to contain less than 5 percent residual N-acetyl groups.

Please replace the paragraph starting on page 18, line 13 of the specification with the following amended paragraph:

22 mg of the type 14 N-acryloylated pneumococcal polysaccharide was dissolved in 1.1 ml of Carbonate/Bicarbonate pH 9.5 buffer. Tetanus toxoid monomer 22 mg was added to the reaction mixture. The reaction mixture was incubated overnight at 37 °C. The progress of the conjugation was analyzed with a ~~Biologic~~-BIOLOGIC system (Bio-Rad) equipped with a superose 12 column. Conjugation of polysaccharide to tetanus toxoid was indicated by the progressive increase in a peak, monitored by measurement of UV absorbance at 280 nm, eluting in the void volume of the column. After conjugation was complete, the solution was neutralized to pH 7 with 0.1N HCl and then dialyzed against PBS. The conjugate was purified by passage over a 1.6.times.60 cm column of ~~Superdex~~-SUPERDEX® 200 PG (Pharmacia) and eluted with PBS containing 0.01% thimerosal. Fractions corresponding to the void-volume peak were pooled. Carbohydrate and protein content in the conjugate were estimated by the phenol-sulfuric assay of Dubois et al. (51) and the Coomassie assay of Bradford (9).

Please replace the paragraph starting on page 19, line 16 of the specification with the following amended paragraph:

300 mg of E. Coli K1 polysaccharide (K1 PS) was dissolved in 15 mL of 2.0 N NaOH solution to which 150 mg of sodium borohydride was added. The solution was heated at 110 °C for 6 hours, cooled down to room temperature and diluted with a 20-fold volume of

dionized water. After diafiltration through an ~~Amicon~~-AMICON YM3 membrane with deionized water, the solution was lyophilized yielding 255 mg of N-deacetylated K1 PS. H¹-NMR at 500 MHz confirmed that complete N-deacetylation occurred.

Please replace the paragraph starting on page 19, line 23 of the specification with the following amended paragraph:

To a 10 mL deionized water solution containing 250 mg of de-N-acetylated K1 PS, cooled in an ice bath, was added dropwise acryloyl chloride (Aldrich, Milwaukee, Wis.) solution, prepared by combining 1 mL of acryloyl chloride with a 1 mL of dioxane. The pH of the solution was maintained between 7.0 and 10.5 by the addition of 2 N sodium hydroxide solution. After completion of the addition, the pH was raised to 13 and maintained between 12.9 to 13.1 for 1 hour at room temperature. The pH of the solution was adjusted to 9.5 by the dropwise addition of 1 N HCL. The solution was diafiltrated with an ~~Amicon~~-AMICON YM3 membrane in a stircell with deionized water. The retentate was lyophilized to dryness, and the material (N-Acryloyl K1 PS) was stored at in a desiccator in a -20°C freezer. H-NMR at 500 MHz indicated that complete N-acryloylation took place during the reaction.

Please replace the paragraph starting on page 20, line 11 of the specification with the following amended paragraph:

A solution containing 8.4 mg of N-Acryloyl K1 PS and 4.0 mg of recombinant *Neisseria meningitidis* PorB in 0.3 mL of 0.2 M borate, 0.05%-ZwittergenTM ZWITTERGENTM 3,14 (Boehringer Mannheim) pH 9.5 was incubated at 37° C for 3 days. The conjugate was purified by size exclusion chromatography through a ~~Superdex~~-SUPERDEX[®] 200 preparative

grade column, and eluted with PBS containing 0.01% thimerosal. The fractions of uv-280 nm active signal eluting at or close to the void volume of the column were pooled and stored in the refrigerator. The conjugate was analysed for sialic acid and protein content by the resorcinol and Coomassie protein assays respectively.

Please replace the paragraph starting on page 20, line 23 of the specification with the following amended paragraph:

To one ml of rPorB porin solution at a conc of 10 mg/ml in 0.25 M HEPES buffer of pH 8.5 containing 0.25 M sodium chloride and 0.05% ~~zwittergent~~ ZWITTERGENT™ 3-14 was added 0.2 ml of 0.05 M N-succinimidyl 3-[2-pyridyldithio]propionate solution. The solution was mixed well and allowed to sit at RT for one hour. To the solution was added 0.06 ml of 1 M dithiothreitol solution in the same buffer. The solution was again mixed well and allowed to sit at RT for an additional two hours. The solution was diluted with 1.3 ml of 0.25 M HEPES buffer of pH 7.0 containing 0.25 M sodium chloride and 0.05% ~~zwittergent~~ ZWITTERGENT™ 3-14 and loaded onto a ~~Pharmacia~~ PHARMACIA PD-10 desalting column which had been pre-equilibrated with the same buffer. The column was eluted with the same buffer, and eluate was collected and concentrated with an ~~Amicon Centricon~~ AMICON CENTRICON 30 concentrator at 5,000 RPM for one hour. The retentate was collected and the protein concentration determined.

Please replace the paragraph starting on page 21, line 6 of the specification with the following amended paragraph:

To 0.17 ml of thiolated rPorB solution at a concentration of 25 mg/ml from above was added 9 mg of N-acryloylated K1 polysaccharide. The solution was mixed well and incubated in an oven of 37°C for 18 hours. The solution was purified through a ~~Superdex~~ SUPERDEX® 200 column (Pharmacia) with PBS as eluent. UV-280-nm-active fractions eluted at or close to the void volume of the column were combined. Analyses showed that the conjugate contained 25 ug/ml of polysaccharide and 188 ug/ml of protein.

Please replace the paragraph starting on page 22, line 19 of the specification with the following amended paragraph:

Immunoassays: Serum antibody to each polysaccharide conjugate was measured by ELISA. The human serum albumin (HSA) (Sigma, St Louis, Mo.) conjugates used for ELISA assays were prepared by reductive amination. The oxidized polysaccharides were added to HSA followed by reductive amination with ~~NaCNBH₃~~ NaCNBH₃. The conjugates were isolated by gel filtration chromatography, and stored freeze-dried at -70 °C. PS-specific antibody titers were determined by an ELISA as follows. Polystyrene, 96-well, flat-bottom microtiter plates (NUNC-~~Polysorb~~ POLYSORB) (Nunc, Naperville, Ill.) were coated with PS-HSA conjugates in PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.5) at 0.25 µg/well (100 µL/well) by incubating for 1 hour at 37 °C, followed by a ~~PBS-Tween~~ PBS-TWEEN® (0.05% v/v ~~Tween~~ TWEEN® 20 in PBS) wash (5 times). All subsequent incubations were conducted at room temperature. ~~PBS-Tween~~ PBS-TWEEN® was used for all required washes. The coated plates were then blocked with PBS-BSA (0.5% w/v bovine serum albumin in PBS) for IgG ELISAs or 0.1% w/v ~~Carnation~~ CARNATION® nonfat dry milk for IgM ELISAs at 0.15 mL / well for 1 hour, followed by a wash. Sera were diluted 2-fold, in duplicate, in the plate at 100 µL/ well and

incubated for 1 hour, followed by a wash. Antibody conjugate (peroxidase-labelled goat anti-mouse (Kirkegaard & Perry Lab, Gaithersburg, Md.) was added at 100 μ L/ well and incubated for 30 minutes, followed by a wash. A 1:1 dye and substrate solution (Kirkegaard & Perry TMB) and peroxide was added at 0.05 mL/ well and incubated for 10 minutes. The peroxidase reaction was then stopped with 1 M H_3PO_4 at 0.05 mL/ well, and the plate was read on a ~~Molecular Devices-Emax~~ MOLECULAR DEVICES EMAX microplate reader (Molecular Devices, Menlo Park, Calif.) at a wavelength of 450 nm, using 650 nm as a reference wavelength. Background absorbances were determined in several no-serum control wells and averaged for each plate. For each serum dilution, the average background absorbance was subtracted, and then duplicate serum absorbance values were averaged. A modified Scatchard plot was used for the subsequent data analysis, where the absorbance (y-axis) was plotted against the absorbance times the reciprocal dilution (x-axis) (ref). Under conditions allowing equilibrium and antibody excess, a straight line was obtained for each serum dilution series; this line was extrapolated to the x-axis for the determination of an antibody titer. A positive control serum, with a previously determined antibody titer, was used on each plate in order to provide a reference to which all sera were standardized, minimizing plate to plate and day to day variations. The results of these assays are shown in Tables 5, 6 and 7.

Please replace the paragraph starting on page 24, line 3 of the specification with the following amended paragraph:

Serum bactericidal assay (SBA): Antibody-dependent complement-mediated bactericidal activity was measured in terms of the bactericidal titer, or reciprocal dilution, that provided 50% killing of the targeted bacteria. The complement in all sera was first incubated at

56 °C for 30 min. Then a 2-fold dilution series was established for each serum with GBSS in sterile 96-well U-bottom microtiter plates (Sigma), giving a final volume of 50 µL/ well. Infant rabbit serum complement (Pel-Freez, Brown Deer, Wis.) was diluted 1:1 with the working concentration of Group B meningococcal (GBM) bacteria (serotype 15 strain, 44/76) or Group C meningococcal C11 reference strain and 50 µL was added to each well containing the diluted serum, giving a final reaction mixture volume of 100 µL/ well. This reaction mixture, which contained 50% serum (heat-inactivated and diluted), 25% rabbit serum complement, and 25% bacteria (at working concentration), was incubated in a humidified incubator at 37 °C with 5% CO₂ for 60 min on a microtitration plate shaker (LKB-Wallac; ~~pharmacia~~-Pharmacia Biotech) at the fast speed.